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# Bone histomorphometry of the clavicle in a forensic sample from Albania

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## Highlights

- Age estimation on the clavicle using histomorphometry has been only performed on four populations.
- None of the existing histological methods provided accurate age estimates for the Albanian sample.
- Population-standards for estimating histological age on the sample under study is deemed to be feasible.

- Bone remodelling differences between Albanian and Americans are reported.


## Abstract

Forensic assessment of skeletal material includes age estimation of unknown individuals. When dealing with extremely fragmented human remains that lack macro-features used in age estimation, histological assessment of the skeletal elements can be employed. Historically, microscopic methods for age assessment used by forensic anthropologists have been available since 1965.Several skeletal elements have been used for this purpose. Among them, the clavicle has garnered very little attention. The purpose of this study is to explore the validity of claviclularhistomorphometry as an age marker in a modern Balkan sample.

This study examined a modern clavicular autopsy sample from Albania. The sample consisted of 33 individuals of known age and cause of death. Data were collected for micro-anatomical features including osteon population density (OPD) and cortical area. Intra- and inter-observer errors were assessed through technical error of measurement (TEM) and *R* coefficient. A validation study was performed in order to test the accuracy of existing histological formulae. Regression analysis was run to developed age prediction models with the best models tested through cross-validation and the comparison between OPD for the Albanian sample and a European-American sample examined.

Intra- and inter-observer error TEM results demonstrated values falling within the limits of acceptance. The existing histological methods did not perform accurately on the sample under study. Regression equations for Albanians produced age estimations deviating 8 and 11 years from known age. Cross-validation on the most accurate regression formula which includes OPD as a single variable demonstrated similar mean errors.Statistically significant differences were observed between the Albanian and the European-American population when the two samples were compared.

The research presented is the fifth article published and the fifth population explored on clavicular microstructure. The potential of histology to estimate age on the Albanian population is shown here; however, population effect, diet and health status might be considered. Further inclusion of individuals will corroborate our preliminary findings.


**Key words**: clavicle, age estimation, forensic anthropology, OPD, Osteons

## Introduction

During a typical forensic examination of human skeletal remains, age estimation is one of the anthropological assessments to be performed for building the biological profile of an unknown individual. Age estimation methods used depend on skeletal elements available for consideration. Macroscopic methods such as the observation of degenerative changes on the auricular surface [1]and on the pubic symphysis [2,3], ecto-cranial suture closure rates [4] or the ossification of the sternal end rib cartilage [5] are also commonly applied. An alternative approach to age assessment relies on the concept that bone remodelling produces micro-anatomical features such as secondary osteons during tissue repair.The accumulation of these micro-features occurs during a mechanism referred as bone turnover rate related to age-specific bone replacement, and barring metabolic influences, the rate of secondary osteon production during a lifetime is predictable [6]. Scholars agree that the amount of secondary intact and fragmentary osteons that form per unit area increases in number as age increases based on the mentioned remodelling mechanism by which cortical bone repair and maintenance occurs throughout one's life [6–9].

Bone histomorphometry is as reliable and feasible for age estimation as macroscopic methods[10]. There have been a number of studies that have tested the accuracy of histological age assessment methods [11], and some disadvantages in forensic applications have been noted. First, assessment of bone micro-anatomy is a destructive and time-consuming process. Second, the costs of the equipment and safe use of chemicals can be problematic for many anthropologists [12]. Third, accurate results require well-trained dry-bone histologists[13–15]. Yet, when there is no appropriate skeletal material available for a specific method or the remains are damaged due to taphonomic effects such as scavenging and erosion, histological methods can still provide accurate age estimation in forensic anthropology casework[16].

Several studies have applied quantitative bone histology in the past to predict age by using different skeletal elements [8,17,18]. Only a few of these methods have withstood the test of time and critics, for example Kerley's[6] femoral equation using intact secondary osteons.The rib has gained more interest compared to other bones mainly due to its smaller cortical area and easy access to sampling[16]. However, bones like the clavicle have not been sufficiently studied at the microscopic level. Stout and Paine's [16] was the first study that used a mixed ethnicity sample of 40 adults to test the clavicle for histological age estimation in isolation and in combination with ribs.In this study, they used a sample of autopsy clavicles from individuals with an age range of 13- 62 years. The variables under consideration were cortical area, complete and fragmented secondary osteon numbers as well as the frequency of secondary osteons per unit of cortical area (Osteon population density, OPD). The study provided simple and multiple logistic regressions to estimate age reporting the highest accuracyfor the multifactorial approach.

Applying Stout and Paine [16] clavicle regression equation, Stout et al. [19] studied 83 clavicles from a Swiss and a European-American sample predicting age within 5.5 years of absolute difference. The reported difference between the mean known age and mean predicted age was attributed to intrinsic population differences and sample demographics, as well as limitations on the applicability of histological age estimation on old individuals due to osteon asymptote. A new age estimation formula was created by the authors to further represent bone remodeling variation on both populations [19].

A more recent study conducted by Lee and colleagues[20]investigated the development of a population-specific formula for a Korean sample (N=46), with an age range of 20-90+ years at death.OPD, mean osteon area and relative cortical area were assessed on eight sampling areas on the periosteal portion of the clavicle with the former parameters positively correlated with age while relative cortical area presented an inverse correlation. Multiple stepwise regression analysis produced an age estimation formula including OPD and relative cortical area with an error from known age of 11 years. Sobol et al. [21] performed bone histology on a Polish autopsy sample testing different cross-sections along the length of the clavicle. Bone mid-shaft was chosen as a preferable sampling location due to lower biomechanical influences and four sub-periosteal fields were assessed producing an $R^2$ranging from 0.82 to 0.33 for single variables.

The handful number of histological studies carried out on clavicles [16,19–21]and the limited number of populations studied (Americans, Swiss, Koreans and Polish) creates the need for further analysis of histomorphometric parameters on this skeletal element[22]. Moreover, possible bias introduced by small sample size, sex distribution, genetic and/or pathologic influences on bone microstructure in relation to the collar bones need additional consideration. Therefore, this study aims to test existing histological methods on individuals from Albania and to explore the relationship between age and the histomorphometric parameters. Previous studies on bone histomorphometry have questioned the applicability of histological methods on non-related samples stressing the need of the development of population-specific formulae[8,23,24].In addition, a comparison of histological values from aEuropean-Americansample will be performed to explore population differences in bone remodeling rates. Overall, this research will offer additional information to the existing literature for clavicular histomorphometry.

## Material and Methods

A total of 33 left clavicle mid-shaft cross-sectional samples were obtained from routine autopsies conducted at the Institute ofForensic Medicine in Tirana (Albania) between 2014 and 2015.

The sample comprised 21 males and 12 females with ages ranging from 12 to 59 years old and a mean age of 40.06 years old (SD=12.94). Clinical history of the individuals revealed pathologies related mostly to atherosclerosis and fat cells observed histologically for 13 individuals. A detailed list of the cause of death for the sample can be found in **Table 1**. Following Stout and Paine's [16] original research on clavicles, the left clavicle was chosen for consistency with previous data collected in order to create compatibility histological data. Sampling only left clavicles also helps to avoid issues with the biomechanics of bilateral asymmetry. Moreover, the clavicle is more advantageous than other skeletal elements such as femur due to its location in the human body providing easy extraction and minimal damage during autopsy and its exclusion in osteometric analysis [25].

**Table 1.** Reported cause of death for the autopsy sample

| |
|---|
| Gunshot wound (3) |
| Car accident (10) |
| Asphyxia (1) |
| Sudden death-cardiac infarction (5) |
| Fire incident (1) |
| Self-poisoning (3) |
| Fall from height (2) |
| Hanging (1) |
| Calcium oxide (1) |
| Drowning (1) |
| Non-violent death (3) |
| Unknown (2) |

The histological preparation of modern dry bones used in this study followed the protocol developed byGarcía-Donas et al.[26]. The observation of the histological parameters was carried out under Reflected Light Microscope (RLM). Each sample was placed on the microscope under reflected light with 4x/0.10 magnification. Leica Application Suite (LAS)® Version 4.6.0 was used to obtained microphotographs. A scale of 500 microns was set and consecutive microphotographswere taken with one third of the surface overlapping. Finally, all images were stitched together and the whole section was made visible on the screen and saved as one file and used for the qualitative and quantitative analysis (**Figure 1**).

**Fig. 1** Clavicle thin-section showing the complete cortical area assessed for histological age.

The histological parameters collected for this study consist of:cortical area (Ct.Ar.) and relative cortical area (Ct.Ar/Tt.Ar); intact and fragmentary secondary osteon number (N.On and N.On.Fg, respectively) and total number of osteons (intact and fragments together, N.On.Tt); andOPD (N.On.Tt divided by Ct.Ar),OPD intact (OPDI as N.On. divided by Ct.Ar)) and OPD fragment (OPDF as N.On.Fg divided by Ct.Ar). OPD and related measurements were assessed following the definitions provided by Stout and Paine [16] and García-Donas[27]. Data acquisition was carried out using *ImageJ 1.48*® and Photoshop® CS6.

*Statistical analysis*

Intra- and inter-observer error assessment were conducted by two of the authors (EMand EK).Intra- and inter-observer error was estimated in23 randomly selected microphotographs (field view of 3x2.22 millimetres) for OPD related parameters that were measured twice by EM (within two months interval) and once by EK. In order to avoid any bias, the blind scoring was carried out without previous knowledge of age, sex or pathology of the individuals. Technical Error of Measurement (TEM), relative TEM (rTEM) and the coefficient of Reliability ($R$) were used to quantify error measurements [28].

Three published equations for age estimation using the clavicle [16,19,20] were applied on the Albanian sample. The estimated ages were calculated following the methods outlined by each study. The parameter values were inserted into the published equations and minimum, maximum and mean errors were calculated.

The Albanian dataset was tested for normality using skewness, kurtosis and through Shapiro-Wilks tests. Pearson's correlation coefficient was used to explore the correlation of the variables and age. Linear regression analysis (LR) was used to generate prediction models for age estimation calculating the standard error of estimate (SEE) and $AIC_c$ and BIC cross-validation indicators to assess the fitness of eachmodels[29]. Independent student's T-test with bootstrapping at 95% confidence to overcome small sample size issues was used to explore differences between the current sample and the sample used by Stout and Paine [16]. In order to analyze the relationship of age with respect to the rest of the significant factors in the data set, a series of linear regression analyses were performed. Leave-one-out cross-validation (LOOCV) approach was run in order to validate the generated models. Statistical analysis was carried out using R and SPSS22.

## Results

*Intra and Inter-observer error*

Inter- and intra-observer errors as estimated using TEM, rTEM and R can be seen in **Table 2**. The rTEM values are below the 5% threshold indicating high degree of agreement between the observers. For all variables, *R* values are over 95% demonstrating that, overall, repeatability of the parameters under study was achieved for both intra- and inter-observer scores.

**Table 2.** Intra- and Inter-observer error

| Intra-Observer Error | | | |
|---|---|---|---|
| **Variable** | **TEM** | **r TEM** | **R** |
| Ct.Ar | 2.75 | 2.09 | 0.99 |
| Ct.Ar/Tt.Ar | 2.85 | 5.28 | 0.95 |
| N.On.Tt | 1.7 | 1.15 | 0.99 |
| OPD | 0.54 | 0.06 | 0.95 |
| **Inter-observer Error** | | | |
| **Variable** | **TEM** | **r TEM** | **R** |
| Ct.Ar | 5.39 | 4.03 | 0.98 |
| Ct.Ar/Tt.Ar | 2.11 | 3.95 | 0.97 |
| N.On.Tt | 3.4 | 2.26 | 0.98 |
| OPD | 0.47 | 0.05 | 0.96 |

*Descriptive statistics and correlation with age*

Descriptive statistics for the sample under study and the assessed variables can be seen in **Table 3.** All variables approximated normal distribution with skewness and kurtosis between -1 and 1 and normality test p-values above 0.05.

**Table 3.** Descriptive statistics of the Albanian sample.

| N=33 | Minimum | Maximum | Mean | Std. Dev. |
|---|---|---|---|---|
| **Age** | 12 | 59 | 40.06 | 12.94 |
| **N.On** | 370 | 1477 | 855.61 | 39.74 |
| **N.On.Fg** | 67 | 831 | 367.15 | 36.47 |
| **N.On.Tt** | 591 | 2061 | 1222.76 | 66.37 |
| **OPDI** | 2.19 | 10.28 | 6.86 | 0.35 |
| **OPDF** | 0.55 | 5.84 | 2.70 | 0.23 |
| **OPD** | 3.49 | 15.16 | 9.56 | 0.44 |
| **Ct.Ar** | 63.03 | 227.56 | 132.65 | 7.12 |
| **Ct.Ar/Tt.Ar($mm^2$)** | 23.59 | 81.74 | 51.53 | 2.57 |

Std. Dev.= Standard Deviation

Age was found to be positively correlated with all variables with the exception of cortical area andrelative cortical area for which a weak negative correlation was observed.Age presents a statistically significant positive correlation with N.On ($r=0.424$, $p=0.001$), N.On.Fg($r=0.456$, $p=0.014$) and N.On.Tt($r=0.505$, $p=0.003$), as well as OPD ($r=0.779$, $p<0.001$), OPDI ($r=0.541$, $p=0.001$)and OPDF ($r=0.675$, $p<0.001$).

*Validation of histological formulae*

The results from the validation study indicate an underestimation of the age of the Albanian individuals with a mean error of over 20 years **(Table 4)**. Stout and Paine [16] formula resulted in the lowest mean absolute error providing the most accurate age estimates for the sample under consideration while Lee et al. [20] formula performed the worst (**Table 4**).

**Table 4.** Age estimation formulae, sample, $R^2$ and Standard Error of Estimate (SEE) reported by the original studies and minimum, maximum and average error obtained for the Albanian sample.

| | Original studies | | | Validation for the Albanian sample | | |
|---|---|---|---|---|---|---|
| **Original study** | **Formula Sample** | **$R^2$** | **SEE** | **Min. Absolute Error** | **Max. Absolute Error** | **Mean Absolute error** |
| *Stout and Paine (1992)* | $Ln_{age}=0.07028 * OPD + 2.216$ N=40, Mixed Americans | 0.69 | 3.57 | 0.30 | 39.6 | 21.8 |
| *Stout et al. (1996)* | $Ln_{age} =0.085 * OPD + 2.033$ N=83, US and Swiss | 0.85 | 2.05 | 1.70 | 40.3 | 23.1 |
| *Lee et al. (2014)* | $Age = 1.412 - 0.282 * RCA + 2.519 * OPD$ N=46, Korean | 0.63 | 11.40 | 8.60 | 45.0 | 28.6 |

Min=Minimum, Max=Maximum

The estimated ages obtained through the application of the three existing histological formulae were plotted against known ages and LR of estimates versus known age were generated. As seen in **Figure 2**, the estimates produced by Lee et al. (2014) deviated considerably from the estimates obtained through Stout and Paine (1992) and Stout et al. (1996).

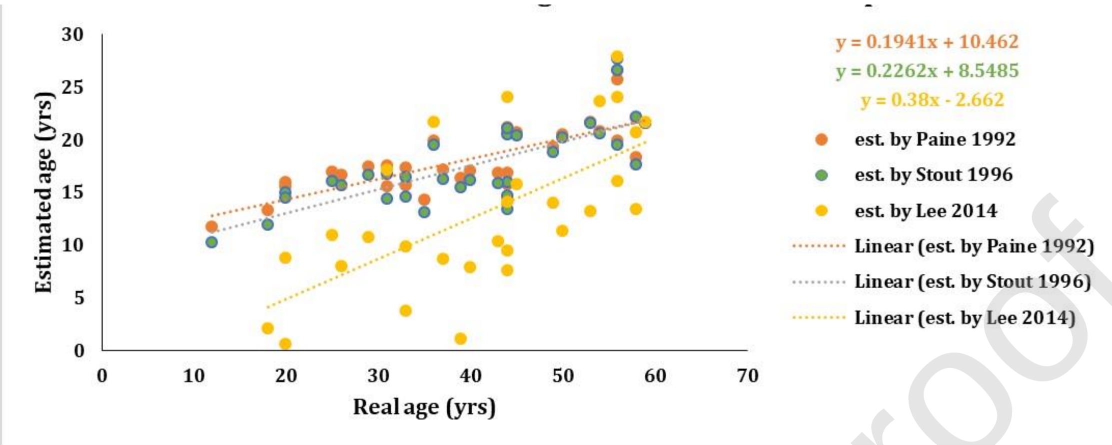



**Fig. 2** Estimated versus known age for the Albanian samples using the formulae developed in previousstudies for different populations; est.= estimated age, yrs=years.

*Age estimation and histological variables*

**Table 5** summarises the regression analysis presenting $R^2$, SEE, AICc and BIC as well as the intercept and coefficient for each model. Taking under consideration the reported values for each formula, R6 provides the most accurate prediction of age for the total sample with $R^2$=0.60 and SEE=8.25 years and the lowest AICc and BIC values indicating the best fit for the data and the generated model (**Figure 3**).

**Table 5**. Simple linear regression for the six statistically significant variables.

| Independent variable (x) | Equation | $R^2$ | F value | Pr> \|t\| | SEE (years) | $AIC_c$ | BIC |
|---|---|---|---|---|---|---|---|
| **R1** | Age = 19.489 + 0.024 x N.On | 0.18 | 6.80 | 0.14 | 11.90 | 261.89 | 265.55 |
| **R2** | Age = 29.713 + 0.028 x N.On.Fg | 0.21 | 8.53 | 0.08 | 11.69 | 270.63 | 264.39 |
| **R3** | Age = 19.119 + 0.017 x N.On.Tt | 0.25 | 10.59 | 0.003 | 11.35 | 258.73 | 262.39 |
| **R4** | Age = 16.021 + 3.504 x OPDI | 0.29 | 12.83 | 0.001 | 11.05 | 257.01 | 260.67 |
| **R5** | Age = 22.288 + 6.578 x OPDF | 0.45 | 25.92 | < 0.001 | 9.70 | 248.38 | 252.04 |
| **R6** | Age = 2.133 + 3.966 x OPD | 0.60 | 47.72 | < 0.001 | 8.25 | 237.68 | 241.35 |

SEE=Standard Error of the estimate

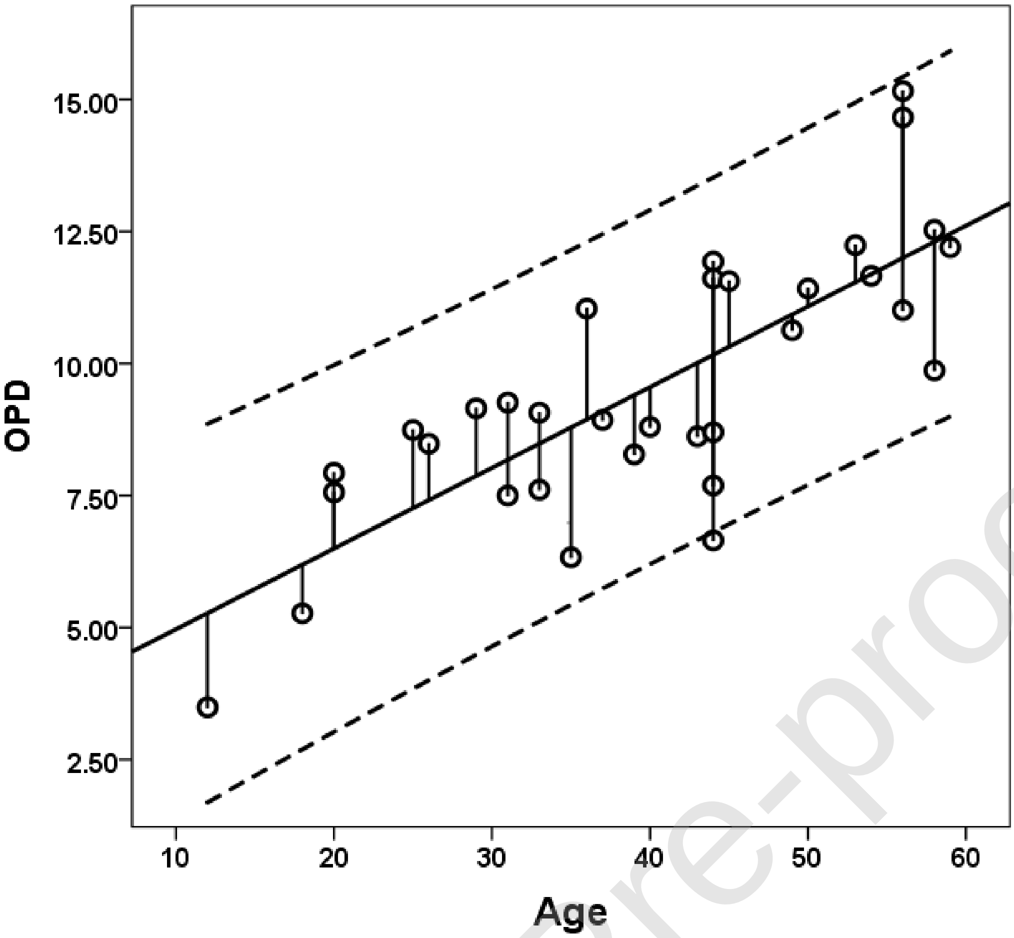
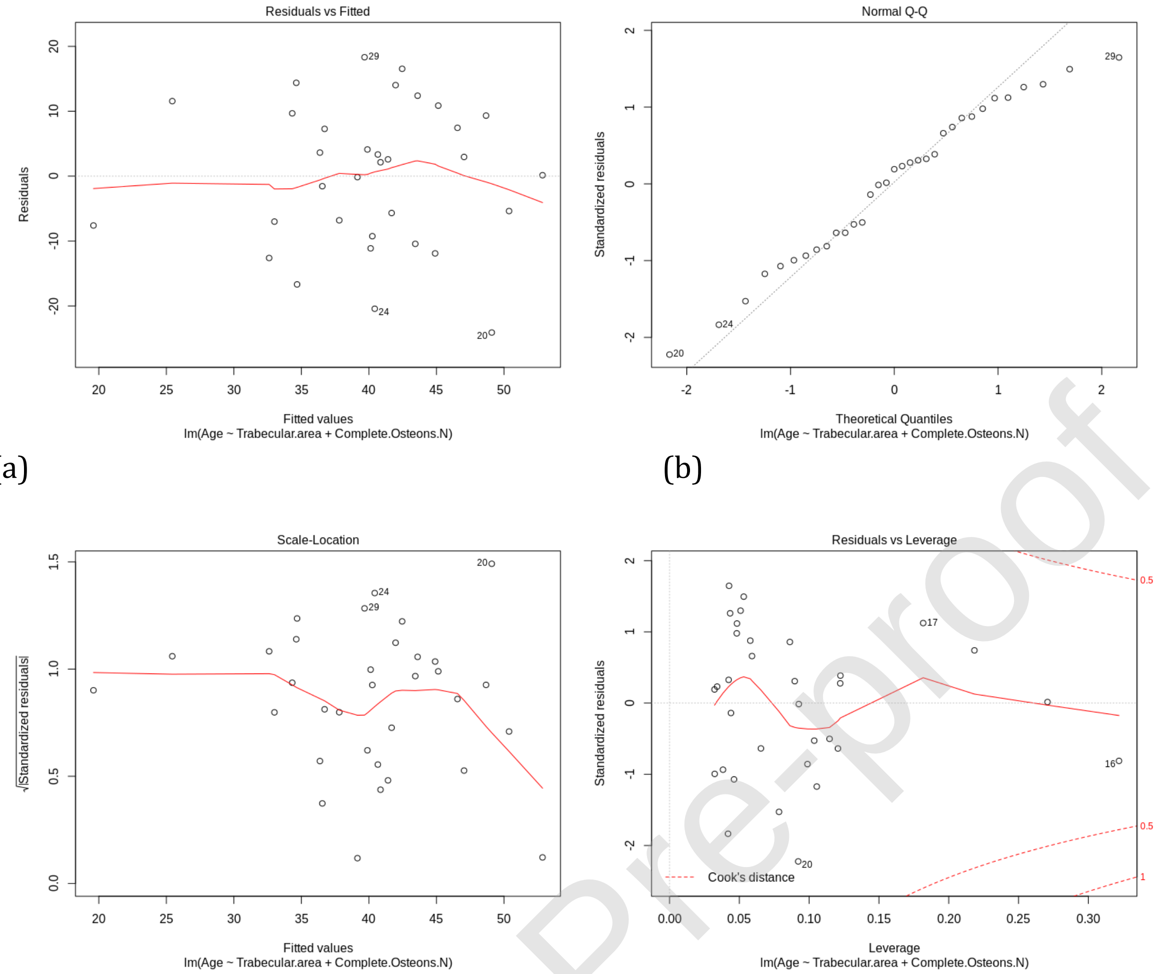



**Fig. 3.** Bivariate plot of OPD and Age for the Albanian data ($R^2$=0.606) showing 95% confidence intervals around the regression line.

Considering the small number of independent variables and individuals, all the formulae presented were tested for a combination of maximum three independent variables. In order to balance the influence of the independent variables, the absolute prediction errors were normalized by dividing each input variable by its mean. As expected, no changes on the prediction errors are observed for single variables (e.g. OPD) after normalization. We automatically produced and tested two dimensional relations. The inclusion of two independent variables in the analysis produced 90 formulae derived from 10 possible meaningful combinations as age and case were removed. All linear regression formulae were also dynamically tested by applyingLOOC. The most accurate age prediction formulae were produced, as reported by the mean absolute errors, by the combination of N.On.Tt and Tb.Ar (SEE=6.54) and N.On.Fg and Tt.Ar (SEE=10.3) for normalized and non-normalized data, respectively. Furthermore, the inclusion of three independent variables was also tested by producing 720 different formulae, yet the prediction accuracy did not show any improvement. Although normalized data produced again better age estimates, no further analysis was performed as the model fit would be compromised by the small sample size. **Figure 4** (a-d) presents the diagnostic plots for the two-independent-variable model that uses normalized data.




(a) (b) (c) (d)

**Figure 4.** Diagnostic plots for lm(Tb.Ar and N.On.Tt) (a) Residuals versus fitted values, (b) Normal Q-Q plot, (c) Spread location plot and (d) Residuals versus leverage.

As aforementioned, R6 provides the most accurate prediction of age for the total sample with $R^2$ of 0.60 and SEE of 8.25 years and the lowest AICc and BIC values indicating the best fit for the data for the generated model. **Figure 5** presents the predicted age (x-axis) and known age (y-axis). The x-axis illustrates R6 SSE (Error 1) and the absolute SEE after LOOC (Mean= 8.24 years) which produced similar outcomes.

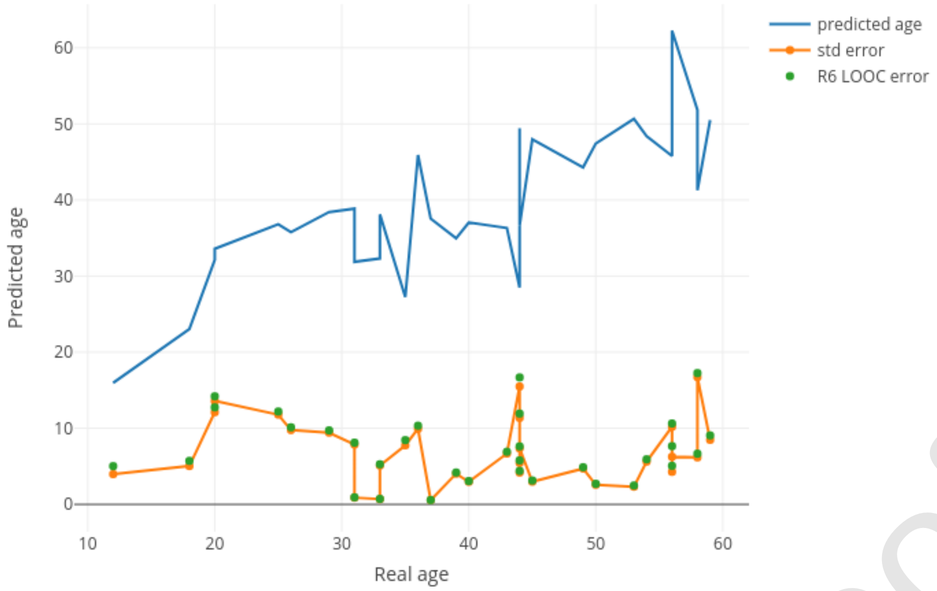



**Figure 5.** Comparison between predicted age and known age for R6: predicted error (blue line), standard error 1 (linear regression-orange line) and standard error 2 (LOOC-green dots).

*Linear regression analysis normalized and non-normalized data and LOOC analyses can be reviewed in Supplementary material 1:*http://forensic.med.uoc.gr/paperAlbanianClavicle.jsp

*Comparison of remodelling rates with other populations*

To explore remodelling rates between different populations and the Albanian sample, the histological data were compared with 35 individuals from American European origin studied by Stout and Paine[16]. The original publication included 40 individuals but five were excluded from this research due to unknown or non-Caucasian ancestry.The data were plotted against real age producing the best fit regression lines as shown in **Figure 6**.

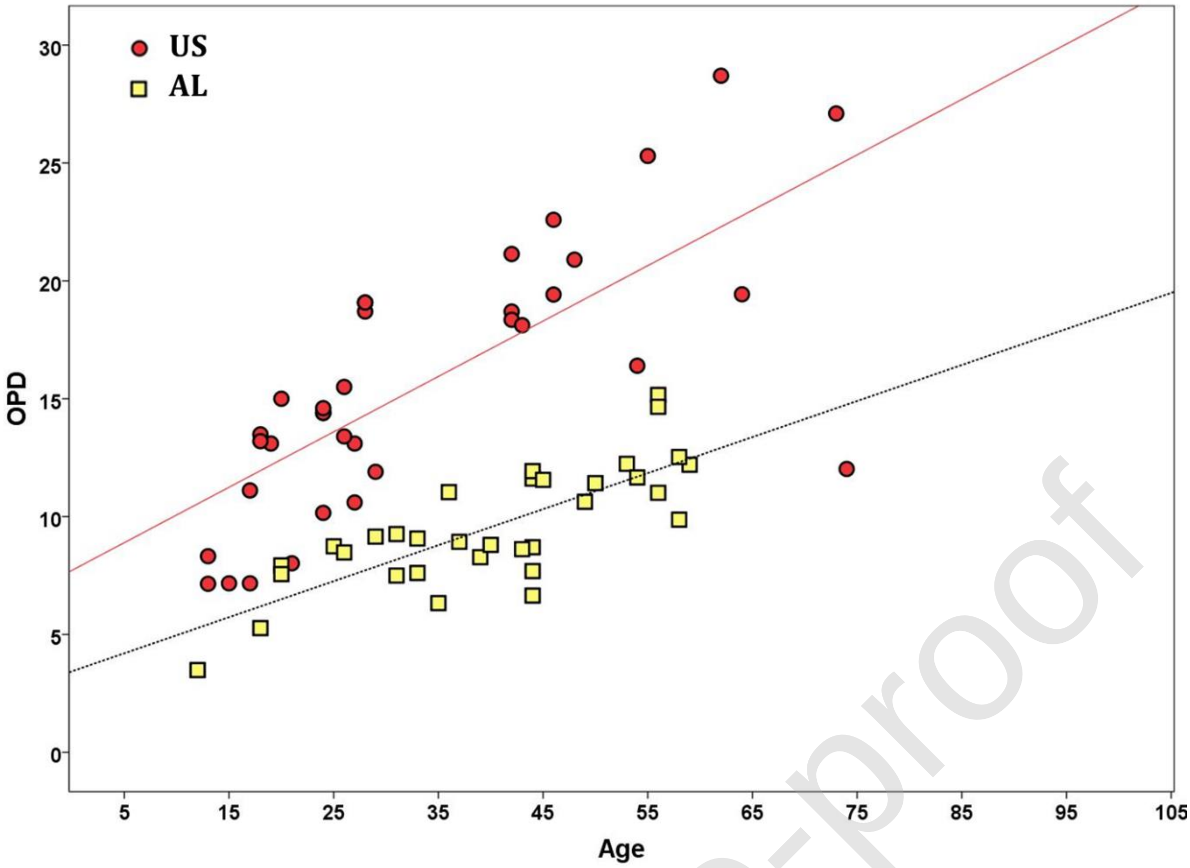



**Fig 6.**Comparison of regression lines between Albanians (AL) and European-Americans (US) [16].

An independent t-test (with 1000 bootstrap samples) was used to analyse the difference between OPD for Albanian and European Americans showing a statistically significant difference between the two populations (t=-5.868, p<0.001).

In order to test the differences between OPD values and age groups, the total sample was then divided according to seven age ranges for which individuals were present for both populations (<20, 20-29, 40-49, 50-59). The four age groups were compared as of their OPD values using an independent T-test with bootstrapping.As seen in**Table 6**, the resultsare statistically significant for the first three age groups, while no difference was observed in the mean OPD valuesfor the 50-59 years old group. The biggest absolute difference between the means is observed for the age group of 40-49 years with American-Europeans exhibiting double the value of mean OPD compared to the Albanians.

**Table 6.**T-test results from a comparison of OPD in Albanian and European-American origin for four age range groups.

| Sample | | Age groups | | | |
|---|---|---|---|---|---|
| | | **<20** | **20-29** | **40-49** | **50-59** |
| **Albanians** | **N** | *2* | *5* | *9* | *9* |
| | **Range** | 3.49-5.27 | 7.56-9.15 | 7.68-11.93 | 9.87-15.16 |
| | **Mean** | 4.38 | 8.37 | 9.57 | 12.30 |
| | **SD** | 1.25 | 0.63 | 1.90 | 1.67 |
| **European Americans** | **N** | *9* | *14* | *7* | *2* |
| | **Range** | 7.15-13.49 | 8.02-15.5 | 18.12-22.59 | 12.02-27.1 |
| | **Mean** | 10.35 | 14.13 | 19.88 | 20.85 |
| | **SD** | 2.85 | 3.33 | 1.68 | 6.29 |
| | **Difference of Means** | | | | |
| | **t-value** | -4.58 | -6.16 | -11.27 | -1.90 |
| | **p-value** | 0.016 | 0.002 | 0.001 | 0.13 |

## Discussion

Age estimation constitutes one of the assessments that the forensic anthropologist is required to perform for identification of unknown skeletal remains. Several factors as observer experience, skeletal element and single or multiple age indicators, as well as the specific statistical approach need to be considered when deciding the method to be applied[30].

In this research, intra- and inter-observer error fell within the agreement threshold as shown by *r*TEM analysis. Several studies have reported high degree of repeatability in histological parameters mostly for intra-observer error [31] while other demonstrated higher errors for inter-observer scores[13]. In order to safeguard the method reliability, errors between observers should be reported in the published literature and different statistical approaches used to further explore bias and inaccuracy between observations.

Three known formulae developed from a mixed ethnicity European American sample [16], a mixed ethnicity North American and Swiss sample [19] and a Korean sample [20] were applied on the Albanian population. The equation developed by Stout and Paine [16] performed slightly better than the others. Yet, the standard errors of the original studies were significantly higher as seen in **Table 2**. In addition, all three methods tested in the current study had a better performance for younger individuals than for older specimens (**Figure 2**) which is in accordance with the findings reported by other studies [32].

Other validation studies have been conducted on different skeletal elements concluding that the histological methods were not adequate for the samples under consideration[33,34]. In relation to Southern European populations, a mixed sample of modern Cretan and Greek-Cypriot ribs was used to test four published histological formulae for age estimation [8,16,31,35] demonstrating a gradual increase of inaccuracy and bias values with increasing age except for Goliath and colleagues' method that performed inversely[27].Although a larger Balkan sample is required to verify our preliminary results, the inadequacy of the existing methods for forensic age estimation on the Albanian population is suggested. Thus, their application in forensic settings might not be appropriate as shown by other studies indicating the necessity of histological population-specific methods [34,36,37].

Based on the reported results, population-specific formulae for the Albanians were developed. Linear regression analysis was performed for each one of the six variables correlated to age (N.On, N.On.Fg, N.On.Tt, OPD, OPDI and OPDF). OPD produced the best formula with the lowest standard error (SEE=8.25 years). The standard errors of the best formula for the Albanians is lower than the reported by Lee et al. [20] but higher than Stout and Paine [16] and Stout et al. [19] errors. It is worth mentioning that the Stout et al. [19] sample comprised Stout and Paine [16] sample plus an additional sample from Switzerland, fact that could explain the similarity in the standard error of estimate. These differences could be further attributed to factors such as population and sex differences, and/or extrinsic factors as well as different methodological approaches. Our preliminary results fall within the standard error reported by macroscopic methods [38], with the possibility of being used for highly fragmented or taphonomically affected human costal elements. Moreover, a subset of 24 individuals from the Albanian sample was recently tested for age-estimation based on observed changes on the trabecular bone of the medial end of the clavicle through μ-CT [39]. The results showed that there was a different trend in qualitative trabecular changes before and after 23 to 25 years of age and volumetric tissue mineral density and trabecular number decreased throughout the age ranges span. Further inclusion of individuals to confirm the use of age-related changes in cancellous bone is needed.

This study attempted a direct comparison of Albanians with a European-American population. The mean OPD for the Albanian and European American samples was proved to be significantly different and further division of the samples into age ranges shown statistically significant differences for the first three groups (<20, 20-29 and 40-49 years). These differences in the OPD values could reflect genetic differences between the samples [36] or differences in any of the other influential factors previously mentioned. Apart from the pathological information reported, there is not available data for diet or physical activity for the sample under study. Thus, further conclusions cannot be reached. While it is not possible to fully interpret the reported differences of this comparison, developing population specific formulae for Albanian individuals suggested that histomorphometric methods are more recommended than the existing microscopic methods and that the developed technique can be reliably applied as shown by the degree of agreement within and between observers.

As aforementioned, physical activity could potentially produce differences in the histological variables as it is related to bone remodeling rates activation.

Frost[40]stipulated that exercise increases bone mass as a response to an increase in mechanical strain. Remodeling on long bone diaphysis as a result of exercise was extensively documented [41–43]. Yet, the exact mechanism of how activity influences bone metabolism remains unclear and it is complicated to monitor such changes in vivo and/or assess their potential impact on histological variables through 2D histology. The clavicle is not a weight-bearing bone, and so is not expected to be affected by factors that could influence osteon remodeling rate, such as physical exercise, labor activity or lifestyle [16].

Diet is yet another factor that might influence health in general [44]and bone turnover rate specifically[45]. A validation of Stout and Paine [16] original methodology in a South African sample suffering from dietary deficiencies produced a systematic underestimation of age suggesting a high impact of diet on histological parameters related to bone remodeling. This is in agreement with studies investigating protein deficiency diets that resulted in similar age underestimation [46]. On the contrary, Weinstein and colleagues [47] reported that a high protein diet causes metabolic acidosis which contributes to bone loss and an over estimation of histological age. Moreover, long periods of malnourishing can cause an increase in ketone bodies slowing down bone metabolism. The Albanian population is expected to have a Mediterranean diet rich in olive oil and vegetables with moderate protein and moderate to high lipids consumption and normal levels of alcohol consumption [44]. It is generally understood that considerable alcohol consumption leads to poor nutritional absorption and less food intake. Both would lead to poor nutrition affecting bone turn-over rate. Our autopsy samplescome from a segment of the community with relatively low socioeconomic status hence a higher probability of suffering from malnutrition compared to the average Albanian might be expected. The possibility of underlying dietary deficiencies or low protein diets of the sample population might have had an impact on their biological age as represented by the observed remodeling rates. To support the possible influence in low bone turn over rated as affected by poor diet, our results match findings offered by Paine and Brenton [45]. They also suggest that dietary issues may contribute to under-age estimations. Unfortunately, the current study is unable to test this hypothesis due to insufficient data.

The presence of metabolic disease [47] or trauma [48] is expected to affect bone metabolism and subsequently the histological age estimation. Clinical data was available for the Albanian sample with 13 individuals presenting different pathological conditions. More specifically, these individuals exhibited from slight to generalized fat accumulation in the liver (N=9) with history of alcoholism, coronary disease (N=2), a combination of coronary disease, right ventricular hypertrophy and hepatomegaly (N=1) while one case presented abnormal bone microstructure that could be due to chronic metabolic disease or cancer metastasis (no clinical data available). Previous histological studies have included pathological individuals to develop age prediction equations either consciously or accidently as numerous pathologies will not be discernible through macroscopic or microscopic observation. For example, Goliath et al. [31], Stout and Paine [16] and Stout et al. [19] used samples that included individuals suffering from pathological conditions. The inclusion of pathological samples allows for capturing wider population variation in histomorphometric values [16,49] but also affects the prediction equations. The general underestimation reported for the Albanian sample(which included pathological and healthy samples) when applying the existing histological formulaemight be related not solely to pathological conditions but also to other intrinsic factors such as genetics [34].

The effect of pathology in bone microstructure and subsequently in age estimation methods is a complex topic that needs a thoroughly designed study based on well-documented samples so that safe conclusions can be reached.

## Conclusion

This study is the fifth worldwide reported on histomorphometry of the clavicle and the first presenting data on the Albanian population. The results indicate that previously developed histological methods based on the remodeling rates for clavicular micro-anatomy are not adequate for determining the age of Albanian skeletal remains, clearly under aging these individuals (SEE of over 20 years). Our Albanian population specific formulae generated a SEE of only 8 years. The results offer additional information on clavicular histomorphometry contributing to the currently limited publications on the topic. Further research on a larger sample is needed in order to offer a reliable age estimation method using clavicular secondary osteons suitable for forensic application.

**Ethical approval**: this research approved by the Ethics Committee of the School of History, Classics and Archaeology of the University of Edinburgh (UK), the Albanian General Prosecution Office of the Ministry of Justice and the Institute of Forensic Medicine in Tirana (Albania).

**Credit author statement**

Conception and design of study: EK, RP, JGGD

Data acquisition: EM, KT, AB, BX

Data analysis and interpretation: JGGD, MK, EK, RP

Drafting the manuscript: JGGD, EM, AB, KT, MK

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